MODULATION OF CYTOCHROME P-450 REDUCTASE ACTIVITY IN POPPY PLANTS

FIELD OF THE INVENTION

The present invention relates to the identification and characterisation of a new cytochrome P-450 reductase gene (CPR2) in alkaloid poppy plants and modulation of the expression of the enzyme encoded by the gene. The invention also relates to the provision of poppy plants with an altered alkaloid content or profile, as well as the purification of alkaloids from the poppy plant.

BACKGROUND OF THE INVENTION

The opium poppy *Papaver somniferum* is grown under strict government control for the production of medically useful alkaloids such as the narcotic analgesic morphine, the antitussive and narcotic analgesic codeine, the antitussive and apoptosis inducer noscapine, the vasodilator papaverine, and the stimulant thebaine which is used in the synthesis of strong analgesics such as Oxycodone and Buprenorphine. The alkaloid content of poppy straw (which includes threshed poppy capsules) is the most important parameter in the efficiency of opium alkaloid production. There have been numerous attempts to enhance the yield of alkaloids per tonne of poppy material. The majority of approaches focus on improving agricultural practices and on established methods of conventional breeding to increase cultivation efficiencies and modify the genotype of the opium poppy plant.

The relative content of alkaloids in poppy plants is also of considerable importance, and has an impact on efficiency of processing of the plant material and the ultimate yield and cost of an alkaloid. Usually only one of the many alkaloids produced by a poppy plant predominates in the capsules. In the opium poppy, this is generally morphine which rapidly accumulates after flowering.

The network of reactions, enzymes, cofactors ~~and~~ metabolic intermediates involved in the synthesis of alkaloids in the opium poppy is complex and is thought to be regulated at numerous points. There are also thought to be a number rate limiting steps ("bottlenecks") where limitations in the availability of either substrates, cofactors or enzymes determine which particular branch of the alkaloid synthetic pathways is favoured and therefore, the ultimate alkaloid profile and the type of alkaloid which predominates in the plant.

A class of enzymes known as cytochrome P-450 enzymes are involved in the generation of several intermediates in alkaloid biosynthesis pathways. Unlike mammalian cytochrome P-450 enzymes, the plant enzymes have been less clearly described. However, it is known that plant cytochrome P-450 enzymes are like mammalian proteins in that they are hemoproteins which have a common prosthetic group containing iron and are membrane-bound proteins of the endoplasmic reticulum.

Many cytochrome P-450 enzymes catalyse the transfer of one atom of oxygen to a substrate and so are also referred to as monooxygenases. The reactions are dependent on cytochrome P-450 reductase which provides electrons to the enzymes, and the co-factor NADPH. Cytochrome P-450 enzymes specific to alkaloid biosynthesis have been identified and characterised for the protopine, berberine, benzylisoquinoline, benzophenanthridine, morphinan and monoterpenoid indole alkaloid biosynthetic pathways. The role of cytochrome P-450 enzymes in alkaloid biosynthesis is exemplified by the synthesis of sanguinarine in *Eschscholzia californica*. Of the six oxidative transformations involved in the conversion of (S) - reticuline to sanguinarine, four are thought to be catalysed by these enzymes. The reactions are highly regio- and stereoselective.

Cytochrome P-450 reductase is thought to be a relatively promiscuous enzyme in that a particular reductase species will reduce a range of distinct cytochrome P-450 enzymes. Although there is some promiscuity within species, available data suggests that there is poor transferability of reductases from diverged species. For example, although cytochrome P-450 reductase from insect cell culture and porcine liver has been shown to transfer electrons to heterologously expressed *Berberis berbamunine* synthase, the highest turnover number was achieved with the *Berberis* reductase.

Plant cytochrome P-450 reductases have been purified or enriched from *Catharanthus roseus,* sweet potato, *Helianthus tuberosous* (Jerusalem artichoke), *Glycine max* (soybean) cell suspension cultures, *Pueraria lobata,* and Petunia flowers. cDNA encoding cytochrome P-450 reductase has been isolated from *Vigna radiata* (mung bean), *C. roseus, H. tuberosous* (Accession No. Z26250, Z26251), *Vicia sativa* (Accession No. Z26252) and *Arabidopsis.* In addition, cDNA cloning and heterologous expression in *E. coli* of the *C. roseus* cytochrome P-450 reductase has been reported.

More recently, International Patent Application No. WO 99/11765 in the name of Johnson & Johnson Research Pty Limited disclosed the purification and characterisation of alleles of a gene (CPR1) encoding cytochrome P-450 reductases from *P. somniferum* and *E. californica*

(see also Rosco A., et al., 1997). The overall sequence homology of the two alleles between the plants was 63% at the nucleotide level and 69% at the amino acid level. This compared to an overall sequence identity to other plant cytochrome P-450 reductases of approximately 50% at both the nucleotide and amino acid levels. That application also discloses the provision of transgenic plants from Type II callus generated from hypocotyls excised from *P. somniferum* and transformed with a polynucleotide encoding cytochrome P-450 reductase by inoculation with disarmed *Agrobacterium tumefaciens.* WO 99/11765 further describes over-expression or suppression of cytochrome P-450 reductase to obtain an altered alkaloid profile in the poppy plant.

(S)-reticuline is an intermediate in biosynthetic pathways in *P. somniferum* leading to phenanthrene alkaloids such as codeine and morphine, and phthalideisoquinoline alkaloids such as noscapine. (S)-reticuline is also present in other plants such as *E. californica, Corydalis kava, Fumaria officinalis, Berberis bulgaris* and *Sanguiniara canadenisis,* and has been identified as a precursor of protopine, benzo[c]phenanthridine alkaloids which include sanguinarine, protoberberine alkaloids such as corydaline and berberine itself. As well as being an important precursor for other alkaloids, (S)-reticuline has been shown to accelerate hair growth in cultured hair cells (Biol. Pharm. Bull., 20(5) 586-588 (1997). However, (S)-reticuline is present in source plants at very low concentrations. For instance, it is found in commercial poppy straw at 0.04%, and is present in the opium of *P. somniferum* in only trace amounts (Brochman-Hanssen, E. and Furaya, T., Planta Med. 12, 328 (1964)).

International patent application No. WO 99/35902 in the name of Tasmanian Alkaloids Pty Limited describes random mutagenesis protocols to obtain a modified *P. somniferum* having a higher (S)-reticuline than morphine content. The increase in (S)-reticuline yield was considered to be due to the modification of an enzyme or enzymes which utilise (S)-reticuline as a substrate or are involved in biosynthesis steps immediately leading from (S)-reticuline, such as the berberine bridge enzyme (BBE) and dehydroreticulinium ion reductase.

[±]-reticuline has previously been synthesised by a lengthy and difficult process (*Chem. Abs,* 51, 8116 (1957); Gopinath K. W., Govindachari, T. R., and Viswanathan N., (1959). The synthesis of the (S) form has also been reported by Konda et al.,1975. Whilst effective, the drawback is that only small quantities of the compound are obtained after a long and costly synthetic process. Hence, providing (S)-reticuline by a totally synthetic route is undesirable and impracticable for making substantial quantities of the compound.

SUMMARY OF THE INVENTION

The present invention relates to the isolation and characterisation of a new gene (CPR2) encoding a cytochrome P-450 reductase enzyme from *Papaver somniferum* poppy although the invention extends to related alleles of other poppy plants. The enzyme has been found to have different substrate specificity and/or reductase activity compared to cytochrome P-450 reductase enzymes encoded by alleles of the CPR1 gene of alkaloid producing poppy plants previously characterised in WO 99/35902.

As such, modulation of the expression of the cytochrome P-450 reductase of the invention or variants thereof may allow the alkaloid profile as well as the total alkaloid yield or type of predominant alkaloid synthesised to be differentially modified compared to that obtained by modulating the activity of the previously characterised cytochrome P-450 reductase enzymes encoded by alleles of the CPR1 gene. For instance, suppressing the expression of the cytochrome P-450 reductase enzyme of the invention in *P. somniferum* may result in substantial accumulation of one or more benzylisoquinoline alkaloids such as (S)-reticuline above a normal level of the alkaloid(s) in the plant. This has not been observed following suppression of the cytochrome P-450 reductase enzyme encoded by the CPR1 gene previously characterised in the plant. However, it will be understood the invention is not limited to suppression of the CPR2 gene, and that the invention encompasses the up-regulation of activity of the cytochrome P-450 reductase enzyme encoded by the CPR2 gene.

Accordingly, in one aspect of the present invention there is provided a purified polynucleotide encoding a cytochrome P-450 reductase enzyme of an alkaloid producing poppy plant involved in a step in an alkaloid biosynthesis pathway of the plant and which inhibits accumulation of at least one benzylisoquinoline alkaloid above a normal level of the alkaloid in the plant, or which encodes an active fragment, homologue or variant thereof having enzymatic activity of the enzyme.

Preferably, the alkaloid biosynthesis pathway will be a pathway for the synthesis of a phenanthrene alkaloid and the reductase enzyme will inhibit the benzylisoquinoline alkaloid from accumulating with the phenanthrene alkaloid in the poppy plant.

Hence, in another aspect of the present invention there is provided a purified polynucleotide encoding a cytochrome P-450 reductase enzyme of an alkaloid producing poppy plant involved in a step in a pathway for the synthesis of a phenanthrene alkaloid and which inhibits at least one benzylisoquinoline alkaloid from accumulating with the phenanthrene

alkaloid in the poppy plant, or which encodes an active fragment, homologue or variant thereof having enzymatic activity of the enzyme.

Typically, the polynucleotide will comprise nucleotides 222 to 2318 of figure 1 or a polynucleotide which is degenerate to that nucleotide sequence. Preferred polynucleotides will typically have at least 60% sequence identity with nucleotides 222 to 2318 of figure 1. Most preferably, the nucleotide sequence identity will be greater than 75%, more preferably greater than 90% and most preferably, greater than 95% or 98%.

A polynucleotide of the invention may also incorporate one or more nucleotide sequences selected from the group consisting of transcriptional regulatory control sequences, leader sequences for directing location of the encoded enzyme within the cell or transport of the enzyme to the extracellular environment, and untranslated sequences. Untranslated sequences may for instance, be selected from the group consisting of introns, and 5' and 3' untranslated sequences.

In another aspect there is provided a purified polynucleotide which is substantially complementary to all or a region of a polynucleotide encoding the cytochrome P-450 reductase of the invention or an active fragment, homologue or variant thereof having enzymatic activity of the enzyme.

In still another aspect there is provided a purified antisense polynucleotide which is complementary to all or a region of a polynucleotide encoding a cytochrome P-450 reductase enzyme of an alkaloid producing poppy plant involved in a step in an alkaloid biosynthesis pathway of the plant and the enzymatic activity of which inhibits accumulation of at least one benzylisoquinoline alkaloid above a normal level of the alkaloid in the plant, or which is capable of hybridising with the polynucleotide encoding the enzyme under cellular conditions such that expression of the enzyme is down-regulated.

Such complementary sequences are useful as probes and primers, as antisense agents, and in the design of RNAi (eg. hairpin RNAi) and other suppressive agents for down regulating expression of the cytochrome P-450 reductase enzyme encoded by the CPR2 gene such as ribozymes and the like.

In a further aspect there is provided a recombinant vector incorporating a polynucleotide of the invention. The vector may be a cloning vector or for instance an expression vector for expression of the polynucleotide. In a particularly preferred embodiment, there is provided a recombinant vector incorporating a polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising nucleotides 222 to 2318 of figure 1 or a partial nucleotide sequence thereof;

(b) a polynucleotide which is degenerate to a polynucleotide of (a); and

(c) a polynucleotide having at least 60% sequence identity with a polynucleotide of (a) or (b).

In yet another aspect there is provided a host cell incorporating a recombinant vector or a polynucleotide of the invention.

In another aspect of the present invention there is provided a callus comprising plant cells transformed or transfected with a polynucleotide or recombinant vector of the invention.

In still another aspect there is provided a cytochrome P-450 reductase enzyme encoded by a polynucleotide of the invention, or an active fragment, homologue, variant, derivative or analogue thereof having activity of the enzyme.

There are also provided plants transformed with a polynucleotide of the invention. The transformed plants will typically exhibit up-regulated or down-regulated activity of the enzyme. Preferably, the cells of such plants will express multiple copies of the transfected polynucleotide. Modulated expression and thereby activity of the enzyme may also be achieved by expression of transfected polynucleotide(s) under the control of a promotor which is stronger than the endogenous promotor of the plant. Preferably, down-regulated expression of the reductase enzyme will comprise substantially silencing expression of the reductase enzyme. The down-regulation of enzyme activity may be achieved utilising antisense agents, hairpin RNAi or for instance, other suppressive agents such as ribozymes as described above.

Hence, in another aspect there is a method for providing a transgenic plant, the method comprising:

transforming a plant cell with a polynucleotide for expression of a cytochrome P-450 reductase enzyme of an alkaloid producing poppy plant involved in a step in an alkaloid biosynthesis pathway of the poppy plant and which inhibits accumulation of at least one benzylisoquinoline alkaloid above a normal level of the alkaloid in the poppy plant, or an active fragment, homologue, or variant thereof having enzymatic activity of the enzyme, or with a polynucleotide for down-regulating expression of the enzyme;

culturing the transformed plant cell to produce cultured cells; and

generating the transgenic plant from the cultured cells wherein cells of the transgenic plant contain the polynucleotide.

In another aspect of the present invention there is provided a method for providing an alkaloid producing poppy plant with an altered alkaloid content, the method comprising up-regulating or down-regulating activity of a cytochrome P-450 reductase enzyme of the plant that inhibits accumulation of at least one benzylisoquinoline alkaloid above a normal level of the alkaloid in the plant.

In a further aspect there is a transgenic plant provided by a method of the invention, or seed, reproductive material, progeny or a descendant thereof. In particular, there is provided a transgenic plant having cells transformed with a polynucleotide for effecting expression of a cytochrome P-450 reductase enzyme of an alkaloid producing poppy plant involved in a step in an alkaloid biosynthesis pathway of the poppy plant and which inhibits accumulation of at least one benzylisoquinoline alkaloid above a normal level of the alkaloid in the poppy plant, or an active fragment, homologue or variant thereof having enzymatic activity of the enzyme, or with a polynucleotide for down-regulating of expression of the enzyme, or seed, reproductive material, progeny or a descendant of the transgenic plant incorporating the polynucleotide.

In another aspect there is provided an alkaloid producing poppy plant with an altered alkaloid content obtained by a method of the invention, or seed, reproductive material, progeny or a descendent thereof. The term "reproductive material" is to be taken to expressly include pollen incorporating a polynucleotide of the invention.

The altered alkaloid content achieved by a method of the invention or displayed by a plant provided by the invention may comprise an altered yield or type of alkaloid in the plant or for instance, an altered blend of alkaloids in the plant. For example, down-regulation of the activity of the cytochrome P-450 enzyme encoded by the CPR2 gene or a variant thereof may result in an alkaloid profile which includes one or more benzylisoquinoline alkaloids such as (S)-reticuline, laudanosine, laudanine and codamine that are not usually found in the plant or if present, are normally only in trace or negligible amounts. That is, the alkaloid profile that is obtained may be one that includes alkaloids normally found in the plant as well as one or more additional alkaloids that are not normally present or which are present in greater amounts relative to the original plant or parent cultivar. Alternatively, or in addition, one or more alkaloids normally present in the plant may be absent or be present in a reduced quantity.

Similarly, as the cytochrome P-450 reductase enzyme of the invention has significantly different substrate specificity and/or reductase activity compared to that encoded by alleles of the CPR1 gene, up-regulating expression or activity of the reductase enzyme encoded by the CPR2 gene or a variant allele thereof may alleviate "bottlenecks" in one or more alkaloid biosynthesis pathways in the plant catalysed by cytochrome P-450 enzyme(s) such that the synthesis of alkaloids downstream of those steps accumulate to a greater degree than in the original plant or parent cultivar.

In another aspect there is provided a stand of stably reproducing alkaloid producing poppy plants of the invention having altered expression of the cytochrome P-450 reductase enzyme. By "stably reproducing" is meant the altered expression of the cytochrome P-450 reductase enzyme is a heritable trait which is stably passed from one generation of the poppy plant to the next.

The present invention also relates to the extraction of alkaloids and alkaloid concentrates from the alkaloid producing poppy plants of the invention. An alkaloid may for instance be extracted from the straw or latex of the poppy plant. In the instance an alkaloid or alkaloid concentrate is obtained from latex, the latex may first be substantially dried to produce opium and the alkaloid or alkaloid concentrate then extracted from the opium.

Hence, in another aspect of the present invention there is provided straw, latex or opium of an alkaloid producing poppy plant, wherein the straw, latex or opium have a total alkaloid content of which at least about 8% comprises one or more benzylisoquinoline alkaloids by weight. Preferably, at least about 10%, 15% or 20% by weight of the total alkaloid content will comprise one or more benzylisoquinoline alkaloids, and more preferably, at least about 30% or 40% by weight.

In another aspect there is a method for providing at least one poppy plant alkaloid or an alkaloid concentrate, the method comprising:

harvesting capsules of a transgenic alkaloid producing poppy plant to obtain straw or latex; and

extracting the alkaloid or alkaloid concentrate from the straw or latex;

wherein the alkaloid producing poppy plant has cells transformed with a polynucleotide that up-regulates expression of a cytochrome P-450 reductase enzyme involved in a step in an alkaloid biosynthesis pathway of the poppy plant and the enzymatic activity of which inhibits accumulation of at least one benzylisoquinoline alkaloid above a normal level of the alkaloid in the poppy plant, or an active fragment, homologue, or variant

thereof having activity of the enzyme, or with a polynucleotide which down-regulates expression of the enzyme in the poppy plant, such that the straw or latex thereby has an altered alkaloid content.

In addition, the invention extends to the alkaloid(s) and alkaloid concentrates obtained. Preferably, an alkaloid provided in accordance with the invention will be selected from the group consisting of phenanthrene alkaloids and benzylisoquinoline alkaloids. Most preferably, the alkaloid will be selected from the group consisting of morphine, codeine, codeinone, thebaine, oripavine, morphine, neopinone, (S)-reticuline, laudanine, laudanosine and codamine. An alkaloid concentrate of the invention may comprise one or more of these alkaloids and one or more other alkaloids.

Preferably, the alkaloid producing poppy plant will be a member of the plant sub-family *Papaveroideae.* Most preferably, the alkaloid producing poppy plant will be *Papaver somniferum.*

All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The features and advantages of the present invention will become further apparent from the following description of preferred embodiments of the invention together with the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a nucleotide sequence comprising the coding sequence of the CPR2 gene (SEQ ID No:1);

Figure 2 shows the amino acid sequence encoded by the CPR2 gene (SEQ ID No:2);

Figure 3 shows part of the biosynthesis pathway leading to the synthesis of morphine in *P. somniferum*;

Figure 4 shows the biosynthesis branch pathway for sanguinarine in *E. californica* and *P. somniferum*;

Figure 5 indicates alkaloids for which (S)-reticuline is an intermediate in their biosynthesis;

Figure 6 is a flow diagram outlining a method for the extraction of (S)-reticuline from an alkaloid extract obtained from opium;

Figure 7 shows the design of the binary vector CPR2 hpRNA;

Figure 8 shows thin layer chromatography (TLC) results indicating the alkaloid profiles of latex collected from T_0 CPR2 transgenic *P. somniferum* plants;

Figure 9 shows TLC results comparing the alkaloid profiles of latex collected from T_0 hpCPR1 and T_0 hpCPR2 transgenic *P. somniferum* plants;

Figure 10 shows TLC results indicating the alkaloid profiles of latex collected from T_1 hpCPR2 transgenic *P. somniferum* plants; and

Figures 11A to 11C show reverse transcriptase polymerase chain reaction (RT-PCR) results for T_1 individuals of the *P. somniferum* family 219-10-1.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The isolation and characterisation of the CPR2 gene from *P. somniferum* is exemplified herein. However, it will be appreciated that alleles and variants of the CPR2 gene from other alkaloid producing poppy plants are within the scope of the invention. The identification and cloning of the CPR2 gene provides a means by which the alkaloid content of such plants may be modified to obtain altered alkaloid profiles and more particularly, alkaloid producing poppy plants in which the blend, yield or type of predominant alkaloid(s) is altered.

Expression of a polynucleotide of the invention in poppy plant cells can be achieved using vectors incorporating a DNA insert for intracellular expression of the cytochrome P-450 reductase enzyme or an active fragment, homologue, analogue, or variant thereof, while down regulation of the activity of the enzyme can be achieved using vectors expressing antisense sequences, plus-sense co-suppression, ribozyme or RNAi sequences which are

specific to the CPR2 gene. The expression or suppression of the activity of the reductase enzyme may comprise transient expression or suppression. Expression vectors incorporating the nucleic acid insert can be introduced into poppy cells for expression of the insert extrachromosomally or more preferably, for facilitating integration of the insert into genomic DNA by heterologous or homologous recombination events.

The term "homologue" is to be taken to mean a molecule that has amino acid sequence similarity. The homology between amino acid sequences is determined by comparing amino acids at each position in the sequences when optimally aligned for the purpose of comparison. The sequences are considered homologous at a position if the amino acids at that position are the same. A gap, that is, a position in an alignment where a residue is present in one sequence but not the other is regarded as a position with non-identical residues. Alignment of sequences may be performed using any suitable program or algorithm such as for instance, by the Needleman and Wunsch algorithm (Needleman and Wunsch., 1970). Computer assisted sequence alignment can be conveniently performed using standard software programs such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madison, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3. Typically, a homologue will have an overall amino acid sequence homology of at least about 60 or 70%, preferably about 80 or 90% or greater and most preferably, about 95% or 98% or greater. Homology with the active site of the enzyme will usually be greater than the overall amino acid sequence homology of the homologue and will usually be greater than at least about 80%, and preferably, greater than about 90%, 95% or 98%.

A homologue may be provided by, or the result of, the addition of one or more amino acid residue(s) to an amino acid sequence, deletion of one or more amino acids from an amino acid sequence and/or the substitution of one or more amino acids with another amino acid or amino acids. Inversion of amino acids and other mutational changes that result in alteration of an amino acid sequence are also encompassed. A homologue may be prepared by introducing nucleotide changes in a nucleic sequence such that the desired amino acid changes are achieved upon expression of the mutagenised nucleic acid or for instance, by synthesising an amino acid sequence incorporating the desired amino acid changes.

The substitution of an amino acid may involve a conservative or non-conservative amino acid substitution. By conservation amino acid substitution is meant replacing an amino residue with another amino acid having similar stererochemical and/or chemical properties, which does not substantially affect the conformation or the desired aspect or aspects of

characteristic biological function. Preferred homologues include ones having amino acid sequences in which one or more amino acids have been substituted with alanine or other neutrally charged amino acid residue(s), or to which one or more such amino acid residues have been added. A homologue may also incorporate an amino acid or amino acids not encoded by the genetic code.

The sequence identity of two nucleotide sequences may be determined using the same methodology as for determining homology between amino acid sequences. It will be understood that in the instance RNA and DNA sequences are compared for sequence identity, thymine (T) in the DNA sequence will be considered for the purpose of the comparison to be the same as uracil (U) in the RNA sequence.

By the term "variant" is meant an isoform of a polypeptide or fragment thereof, a naturally occurring mutant form or a polypeptide or fragment thereof or a polypeptide or fragment thereof encoded by an allelic variant or partial nucleic acid sequence thereof. A variant will typically have at least the same degree of homology as a homologue as described herein.

The term "analogue" encompasses a molecule that differs from the original molecule but retains similarity in one or more features that provide the biological function characteristic of the original molecule. An analogue may have substantial overall structural similarity with the original molecule or only structural similarity with one or more regions of the original molecule responsible for the desired characteristic biological function. By "structural" similarity is meant similarity in shape, conformation and/or other structural features responsible for the provision of the biological function or which otherwise have involvement in provision of the biological function.

By the term "derivative" is meant a molecule that is derived or obtained from the original molecule and which retains one or more aspects of characteristic biological function of that molecule. A derivative may for instance result form post-translational or post-synthesis modification such as the attachment of carbohydrate moieties or chemical reaction(s) resulting in structural modification(s) such as the alkylation or acetylation of amino acid residues or other changes involving the formation of chemical bonds.

The metabolic pathway leading to the synthesis of the phenanthrene alkaloids thebaine, neopinone, morphinone, oripavine, codeinone, codeine and morphine in the opium poppy *P. somniferum* is depicted in Figure 3. The steps and intermediates leading to the synthesis of (S)-reticuline are not shown.

Typically, cytochrome P-450 enzymes exist in a 15 to 20 fold excess to the level of cytochrome P-450 reductase enzyme. There is also approximately a 6:1 dependence between the two types of enzyme and it is therefore believed that the level of reductase limits the rate of activity of the cytochrome P-450 enzymes. Supplying plant tissue with radio labelled reticuline and salutaridinol, has been found to result in accumulation of radioactivity at thebaine. Similarly, the addition of radiolabelled compounds after thebaine results in accumulation of radioactivity at codeinone (see WO 99/11765).

Oripavine and morphinone are intermediates in a branch biosynthesis pathway leading from thebaine to morphine, while neopinone is an intermediate in a further alternate biosynthesis pathway from thebaine to morphine. The following steps are known or suspected by the applicant of being catalysed by cytochrome P-450 enzymes which are rate limiting:

(R)-reticuline → salutaridine

Thebaine → neopinone → codeinone

Thebaine → oripavine

Oripavine → morphinone

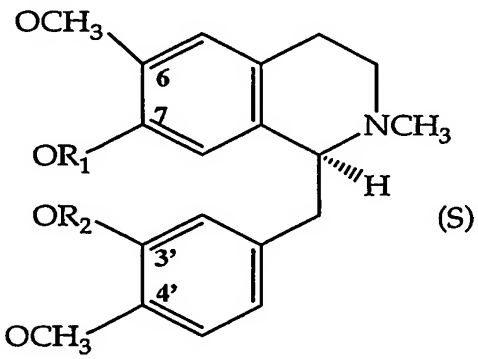
Codeine →morphine

As noted above, (S)-reticuline is also used as a substrate in the biosynthesis of a range of other alkaloids by different metabolic pathways and of the six oxidative transformations involved in the conversion of (S)-reticuline to sanguinarine, four are thought by the applicant to be catalysed by cytochrome P-450 enzymes. The biosynthesis pathway for sanguinarine in *E. californica* is shown is Fig. 4 while alkaloids for which (S)-reticuline is an intermediate in their biosynthesis are illustrated in Fig. 5.

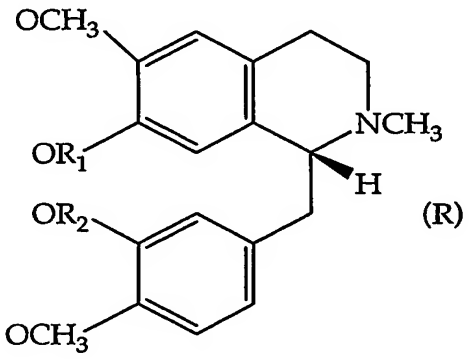
Accordingly, by up-regulating the expression of the cytochrome P-450 reductase enzyme encoded by the CPR2 gene or a variant thereof, one or more of the bottlenecks catalysed by cytochrome P-450 enzymes in poppy plants may be ameliorated, driving the synthesis of alkaloids downstream of such rate-limiting steps forward. For example, in the morphinone biosynthesis pathway, this may result in greater accumulation of one or more of morphine, thebaine, codeine, oripavine or other phenanthrene alkaloid. Similarly, in the sanguinarine biosynthesis pathway, up-regulation of expression of the reductase enzyme may result in

greater accumulation of sanguinarine or alkaloid intermediate(s) in that pathway. Laudanosine, laudanine and codamine have structural similarity with (S)-reticuline but the pathways for their biosynthesis have not currently been elucidated. The structures for these compounds is indicated in Scheme 1.

Scheme 1:



(S)



(R)

		# O-methyls
reticuline	R1 = H, R2 = H	2
laudanine	R1 = CH3, R2 =H	3
codamine	R1 = H, R2 = CH3	3
laudanosine	R1 = CH3, R2 = CH3	4

In one form, broadly stated, the down regulation of activity of the cytochrome P-450 reductase enzyme encoded by the CPR2 gene or a variant thereof may comprise introducing into poppy cells a first nucleic acid molecule that is capable of interacting with the CPR2 gene or variant thereof, or which is capable of being transcribed to a nucleic acid molecule that interacts with the CPR2 gene or a variant allele thereof, such that expression of the CPR2 gene or variant thereof is inhibited.

Reference to the first nucleic acid molecule is to be understood as a reference to any nucleic acid molecule which directly or indirectly facilitates the reduction, inhibition or other form of down regulation of expression of the cytochrome P-450 reductase enzyme. Nucleic acid molecules which fall within the scope of this definition include antisense sequences which have been administered to the poppy cells, and antisense sequences generated *in situ* which have sufficient complementarity with target sequence such as mRNA coding for the enzyme or for instance a transcription regulatory sequence controlling the transcription of the CPR2 gene, to be capable of hybridising with the target sequence under intracellular conditions and thereby inhibit the expression of the enzyme.

The use of chimeric DNA constructs encoding RNA which is capable of forming double stranded RNA (dsRNA) by base pairing between antisense and sense RNA nucleotide sequences which are respectively complementary to corresponding strands of target sequences is particularly preferred. Such dsRNA is also referred to as hairpin RNA (hpRNA) or interfering RNA (RNAi). The design of dsRNA for down-regulating the expression of target nucleic acid sequence has previously been described in International Patent Application No. WO 99/53050 and International Patent Application No. WO 03/076620, as well as for instance, in articles by Wesley S.V. et al., "Construct design for efficient, effective and high-throughput gene silencing in plants". *The Plant Journal* (2001) 27(6): 581-590, and Wang M-B., and Waterhouse P.M., "Application of gene silencing in plants", *Curr. Opp. in Plant. Biol.* (2002), 5(2):146-150, all of which are herein incorporated by reference in their entirety.

More particularly, hpRNA constructs require at least two copies of target sequence in an inverted-repeat orientation which are sufficiently complementary to each other to hybridise together to produce dsRNA. The inverted-repeat sequences will typically be separated by a spacer sequence for forming the end loop region of the hpRNA. The spacer sequence may consist of, or include, an intron sequence that is subsequently spliced out leaving a shorter non-base pairing region that forms the loop. It is not necessary that the inverted repeats of the hairpin RNA be of the same length and one may be longer than the other such that an overhang sequence is produced.

The inclusion of an intron sequence in the chimeric construct encoding the hpRNA, preferably in the sense orientation, may enhance the efficiency of suppression of the expression of the target nucleic acid. Specifically, as used herein, an "intron" or intervening sequence refers to a DNA region between the sense and antisense sequences which is transcribed to yield an untranslated region in the nucleus but which is spliced out in the cytoplasm of a cell. Intron sequences are flanked by splice sites, and synthetic introns may be made by joining appropriate splice sites to any non-coding sequence. Examples of introns include the pdk2 intron, catalase introns from Castor Bean, Delta 12 desaturase intron from *Arabidopsis,* ubiquitin intron sequences from maize, and SV40 introns.

The longer the total length of the sense nucleotide sequence is, the less stringent the requirements for sequence identity between the sense nucleotide sequence and the corresponding target sequence becomes. Accordingly, it is not necessary that the sense nucleotide sequence have total complementarity with its target sequence only that substantial complementarity exists for specificity and to allow hybridisation under cellular

conditions. Preferably, the sense sequence will have a complementarity of about 70% or greater, more preferably about 80% or greater and most preferably, about 90%, 95%, or 98% or greater.

The length of the sense sequence may vary from about 10 nucleotides up to a length equalling the length in nucleotides of the target nucleic acid. Preferably, the total length of the sense sequence will usually be about 15 nucleotides in length or greater, preferably at least about 50 nucleotides, more preferably at least about 100 nucleotides and more preferably at least about 200 nucleotides, 500 nucleotides or at least about 700 nucleotides or 1000 nucleotides in length or greater.

Similarly, the length of the antisense nucleotide sequence is largely determined by the length of the sense sequence and generally, will be the same length as the sense sequence. However, an antisense sequence which differs in length by about 10% or more compared to the sense sequence may be utilised. Similarly, the nucleotide sequence of the antisense sequence is largely determined by the nucleotide sequence of the sense sequence and preferably, is entirely complementary to the sense nucleotide sequence. However, particularly with longer antisense regions, it is possible to use antisense sequences that are not entirely complementary and include some mismatched bases. Preferably, the antisense nucleotide sequence has at least about 75% sequence identity with the sense nucleotide sequence, more preferably at least about 80%, 85% or 90% sequence identity and more preferably at least about 95% sequence complementarity with the sense nucleotide sequence.

Nevertheless, the antisense nucleotide sequence will generally include a sequence of about 10 consecutive nucleotides, more preferably about 15, 20 or 50 nucleotides and most preferably about 100 or 150 nucleotides with 100% sequence identity to the corresponding region of the sense nucleotide sequence.

Alternatively, rather than the inverted-repeats of the hpRNA comprising sense and antisense sequences of the target sequence, chimeric constructs may be designed for the generation of dsRNA comprising a single stranded RNA sequence specific for the target sequence and which is arranged adjacent to a potential hairpin-forming structure incorporating inverted repeats which do not hybridise with target sequence (adj-hpRNA). In this instance, the sequence encoding the hpRNA may be generic to the vector used while the specificity of the suppression is accomplished by the single stranded sequence.

The down-regulation of the CPR2 gene will typically comprise substantially silencing the CPR2 gene. By silencing the gene, one or more benzylisoquinoline alkaloids such as (S)-reticuline, laudanosine, laudanine and/or codamine may accumulate to a level greater than 25%, 50%, 100%, 500% or more preferably, greater than 1000% of the normal level of the alkaloid in the poppy plant.

Nucleic acid sequences for modulating the activity of the cytochrome P-450 reductase enzyme encoded by the CPR2 gene may be generated *in vivo* by transcription of a suitable expression vector within a cell transformed with the vector, or *ex vivo* and then be introduced into target cells. Such nucleic acid sequences will desirably be designed to be resistant to endogenous exonucleases and/or endonucleases to provide *in vivo* stability in target cells. Modification to the phosphate backbone, sugar moieties or nucleic acid bases may also be made to enhance solubility or other physical characteristic, and all such modifications are expressly encompassed. Such modifications include modification of the phosphodiester linkages between sugar moieties and the utilisation of synthetic nucleotides and substituted sugar moieties and the like.

Nucleic acid will typically first be introduced into a cloning vector and amplified in host cells, typically animal, insect or prokaryotic cells, prior to the nucleic acid being excised and incorporated into a suitable expression vector. Typical cloning vectors incorporate an origin of replication (*ori*) for permitting efficient replication of the vector, a reporter or marker gene for enabling selection of host cells transformed with the vector, and restriction enzyme cleavage sites for facilitating the insertion and subsequent excision of the nucleic acid sequence of interest. Preferably, the cloning vector will have a polylinker sequence incorporating an array of restriction sites.

Marker genes used for use in animal or prokaryotic cells may comprise a drug-resistance gene (eg. Amp^r for ampicillin resistance), a gene encoding an enzyme such as chloranphenicol acetyltransferase (CAT), β-Lactamase, adenosine deaminase (ADA), aminoglycoside phosphotransferase (APH), dihydrofolate reductase (DHFR), or for instance β-galactosidase encoded by the *E-coli lacZ* gene (LacZ). Yeast reported genes include imidazole glycerolphosphate dehydratase (HIS3), *N*-(5'-phosphoribosyl)-anthranilate isomerase (TRP1) and β-isopropylmalate dehydrogenase (LEU2).

Marker genes particularly suitable for use in plant cells include but are not limited to visual marker genes such as seed coat colour genes (e.g., the corn R-gene), the gene encoding dihydrofolate reductase (DHFR), sun flower albumin gene *SF8g* which enables a novel

sunflower seed albumin to accumulate in seed, plant-expressable β -glucuronidase genes such as GUS an enzyme that is similar to the E-coli β-galactosidase enzyme but which instead uses glucuronides as substrate, the gene encoding green fluorescent protein (GFP), the *luciferase* gene (the enzyme encoded by the gene catalyses a reaction in which luciferin is oxidised and ATP is converted to AMP, and light is produced which can be measured with a luminometer or detected using photographic film as is known in the art), the *pat* gene which confers Basta herbicide resistance and enables selection of transformed cells using the herbicide or the active ingredient phoshinothricin (PPT), and genes which confer paromomycin, hygromycin, kanamycin or spectinomycin resistance. As will be appreciated, expression vectors of the invention may also incorporate suitable such marker genes.

Suitable expression vectors include cosmids and plasmids such as the Ti- or Ri-plasmids of *Agrobacterium* capable of expression of a DNA (e.g., genomic DNA or cDNA) insert. An expression vector will typically include transcriptional regulatory control sequences to which the inserted nucleic acid sequence is operably linked. By "operably linked" is meant the nucleic acid insert is linked to the transcriptional regulatory control sequences for permitting transcription of the inserted sequence without a shift in the reading frame of the insert. Such transcriptional regulatory control sequences include promoters for facilitating and binding of RNA polymerase to initiate transcription, and expression control elements for enabling binding of ribosomes to transcribed mRNA.

More particularly, the term "regulatory control sequence" as used herein is to be taken to encompass any DNA that is involved in driving transcription and controlling (i.e., regulating) the timing and level of transcription of a given DNA sequence, such as a DNA coding for a protein or polypeptide. For example, a 5' regulatory control sequence is a DNA sequence located upstream of a coding sequence and which may comprise the promotor and the 5' untranslated leader sequence. A 3' regulatory control sequence is a DNA sequence located downstream of the coding sequence, which may comprise suitable transcription termination (and/or) regulation signals, including one or more polyadenylation signals.

As used herein, the term "promotor" encompasses any DNA which is recognised and bound (directly or indirectly) by a DNA-dependent RNA polymerase during initiation of transcription. A promotor includes the transcription initiation site, and binding sites for transcription initiation factors and RNA polymerase, and can comprise various other sites (e.g., enhancers), to which gene expression regulatory proteins may bind.

The promotor may be a constitutive promotor or for instance an inducible promotor the activity of which is enhanced by external or internal stimuli such as but not limited to hormones, chemical compounds, mechanical impulses, and abiotic or biotic stress conditions. The activity of the promotor may also be regulated in a temporal or spatial manner (e.g., tissue specific promotors and developmentally regulated promotors). In a particularly preferred form of the invention, the promotor is a plant-expressible promotor. The term "plant-expressible promotor" is to be taken to mean a DNA sequence that is capable of controlling (initiating) transcription in a plant cell. This includes any promotor of plant origin, but also any promotor of non-plant origin which is capable of directing transcription in a plant cell, such as certain promotors of viral or bacterial origin, e.g., the CaMV35S promotor (Hapster et al., 1988), subterranean stunt clover virus promotor No. 4 or No. 7 (WO 96/06932), and T-DNA gene promotors. Rather than transfecting cells with a polynucleotide sequence encoding the CPR2 cytochrome P-450 reductase enzyme, or an active fragment, homologue, or variant thereof to obtain up regulated activity of the enzyme, cells may simply be transfected with a strong promotor or other transcriptional control sequence for effecting expression of the endogenous polynucleotide encoding the enzyme to a greater degree than then endogeuous promotor or transcriptional regulatory control sequence.

Suitable transcription termination and polyadenylation regions include but are not limited to the SV40 polyadenylation signal, the HSV TK polyadenylation signal, the nopaline synthase gene terminator of *Agrobacterium tumefaciens*, the terminator of the CaMV 35S transcript, terminators of the subterranean stunt clover virus, the terminator of the *Aspergillus nidulans* trpC gene and the like.

Numerous expression vectors suitable for heterologous expression of the cytochrome P-450 reductase enzyme, or fragments, homologues, analogues or variants thereof in prokaryotic (e.g., bacterial) or eukaryotic (e.g., yeast, plant, insect or mammalian cells) are known in the art. Expression vectors suitable for transfection of eukaryotic cells include non-replicating adenoviral shuttle vectors incorporating the polyadenylation site and elongation factor 1-α promotor and pAdEasy expression vectors such as those incorporating a viral promoter (e.g., cytomegalovirus (CMV) promoter). For expression in insect cells, baculovirus expression vectors may be utilised. Viral expression vectors are particularly preferred.

Host cells that may be used for cloning or heterologous expression and subsequent purification of the enzyme include bacteria such as *E. coli*, *Bacillus* such as *B. subtilis*, *Streptomyces* and *Pseudomonas* bacterial strains, yeast such as *Sacchromyces* and *Pichia* cells, insect cells such as *S. frugiperda* Sf9 cells, avian cells and mammalian cells such as

Chinese Hamster Ovary Cells (CHO), COS and HeLa cells. However, of course, plant cells including cells from alkaloid producing poppy plants such as *P. somniferum* or *E. californica* may also be utilised for expression of transfected polynucleotide sequences. The host cells are cultured in a suitable culture median under conditions for facilitating expression of the introduced nucleic acid prior to purification of the expressed product from the host cells, and/or supernatants as the case may be using standard purification techniques.

Any means for achieving the introduction of the nucleic acid into a target cell may be used. Transfer methods known in the art include viral and non-viral transfer methods. For non-plant cells, suitable virus into which appropriate viral expression vectors may be packaged for delivery to target cells include adenovirus, herpes viruses including Herpes Simplex Virus (HSV) and EBV, papovaviruses such as SV40, and adeno-associated viruses. Particularly preferred virus for achieving transfection of animal cells are replication deficient recombinant adenovirus (He et al., 1998). As indicated above, nucleic acid transfer may also be carried out utilising a disarmed Ti-plasmid carried by *Agrobacterium*. Such transformations may for instance be carried out following protocols described in EP 0116718. Plant RNA virus-mediated transformation protocols are described in for example EP 0067553 and US 4,407,956.

Alternatively, vectors incorporating nucleic acid inserts may be intracellularly delivered *in vitro* using conventional cold or heat shock techniques or for instance, calcium phosphate coprecipitation or electroporation protocols. Alternatively, nucleic acid or expression vectors for instance, may be intracellularly delivered by microinjection, microprojectile bombardment utilising particles to which the expression vector or nucleic acid is adhered (Fromm et al., 1990; Gordon-Kamm et al., 1990) or liposome mediated delivery may be utilised. The vector or nucleic acid may be introduced into the host cell(s) with components that enhance nucleic acid uptake by the cell or for instance, stabilise annealed nucleic acid strands.

Vectors and chimeric constructs as described herein may be readily provided by conventional recombinant techniques and be delivered to cells using convention protocols as described in for example, Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Ed., Cold Spring Harbour Laboratory Press, New York; Ausubel et al. (1994) Current Protocols in Molecular Biology, USA, Vol.1 and 2; and Plant Molecular Biology Labfax (1993) by R.D.D. Croy, BIOS Scientific Publications Limited (UK) and Blackwell Scientific Publications, UK.

Once transformation of the plant cells has been achieved, the cells may then be used to regenerate a transgenic plant using protocols such as described in International Patent Application No. WO 99/34663 for the generation of transgenic alkaloid producing poppy plants. In particular, that application teaches that a large rise in the pH in tissue culture media may arise during the transformation of poppy plant tissues or cells such as those of *P. somniferum* in marked contrast to non-poppy plant species. The change in pH may be very rapid and substantial. For instance, pH changes of from 5.6 to greater than 6.4 in the immediate area around a Type II callus in standard B50 medium have been observed within 30 minutes, rising ultimately to pH 8.7. This change in pH has been identified as a major contributor of poor growth and difficulty in producing transgenic alkaloid producing poppy plants. To ameliorate this problem, the pH of poppy plant cell or tissue cultures is desirably maintained within a range of from about pH 5.5 to 6.5 during both transformation of the alkaloid poppy cells and regeneration of transgenic plants.

Preferably, the medium used for transformation and culturing of poppy plants cells and tissues will comprise 19D also known as Callusing Medium (which contains B5 macronutrients, micronutrients, iron salts, vitamins, and sucrose (see Gamborg et al., 1968)) buffered with MES (2-[*N*-morpholino]ethanesulfonic acid. However, any medium modification which maintains the pH within the range of from pH 5.5 to 6.5 may be utilised. MES buffer will usually be utilised in the media at a final concentration of about 10 mM. Alternatively, bis-Tris buffer may be utilised at a concentration of about 10 mM, or the ammonium and nitrate ion amount and ratio in the culture medium selected may be modified. For example, a ratio of nitrate ion to ammonium ion (NO_3^-/NH_4^+) of 1:3 providing a combined nitrogen concentration of 30 mM may be utilised. In addition to helping control the medium pH during culture, the use of buffering agents may produce direct or indirect benefits to the process such as improving *Agrobacterium*-mediated nucleic acid transfer, Type II callus formation, and somatic embryo formation and development. The significance of pH on the transfer of T-DNA by *Agrobacterium* has previously been reported in the art (e.g., Fenning et al.,1996(a) and (b); Li and Komatsuda., 1995).

Protocols for recovering alkaloids from either dried threshed straw or from the opium or latex of poppy plants are well established in the art. For example, a flow diagram outlining the preferential extraction of (S)-reticuline from the alkaloid containing extract of opium is illustrated in Fig. 6 (see Brochmann-Hanssen and Furuya., *Planta Med.* 12: 328-333).

In particular, an acidic extract (pH 1.5) of opium or extracted alkaloid concentrate is obtained in a known manner. The acidic fraction is extracted with chloroform, which removes a number of alkaloids including papaverine, narcotine, thebaine and laudanosine, where present. The acidic aqueous phase is then treated with dichloroacetic acid and further extracted with chloroform. Morphine and codeine remain in the aqueous phase but a number of alkaloids including (S)-reticuline partition into the organic phase. The organic phase is subsequently evaporated to dryness and the residue dissolved in 0.1 M NaOH. Laudanine and laudanidine partition into the chloroform layer. The aqueous layer is treated with sodium bicarbonate and the resultant aqueous solution extracted with ether to obtain a fraction containing (S)-reticuline. Other alkaloids may be extracted from the various fractions obtained using protocol specific for such alkaloids.

However, it will be understood by those skilled in the art that there are a number of suitable starting materials for such extractions depending on the industrial process being used and that Fig. 6 provides one example only. For example, a further technique for the extraction of (S)-reticuline from an alkaloid concentrate obtained from dried ground straw extracted with 80% ethanol and acetic acid at pH 4.5 is described in WO 99/35902. The alkaloids present in an alkaloid extract or fraction obtained by a purification process may be identified by thin layer chromatography (TLC), high pressure liquid chromatography (HPLC), mass spectrometry or other suitable analytical methods. Alternatively, a combination of such methods may be utilised.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following non-limiting examples.

EXAMPLE 1: Isolation and identification of the full length cDNA encoding CPR2 cytochrome P-450 reductase from *P. somniferum*

Poppy RNA from transgenic *E. californica* was screened by PCR for the presence of the CPR1 gene utilising primers designed from the nucleotide sequence encoding the CPR1 cytochrome P-450 reductase enzyme of that plant (Rosco et al., 1997; WO 99/11765). For a negative control, RNA from a wild-type *P. somniferum* (genotype CO48, Tasmanian Alkaloids Pty Limited, Westbury, Tasmania, Australia) was also screened utilising the same primers. RNA was isolated from mature leaves using a Qiagen RNeasy plant minikit, and one step RT-PCR amplification on 100ng of poppy RNA was performed using a Qiagen One Step RT-PCR kit. Surprisingly, a product was observed for the negative control. The specific sequences for the primers used are as follows:

5′ GAAGGAGCTTCACACTCCAGTATCTG 3′ (ECPR 1056F) (SEQ ID No:3)

5′ TCACCACACATCACGTAGATACCTTCC 3′ (ECPR 2241R) (SEQ ID No:4)

The one step RT-PCR amplification was performed using the following conditions for denaturation, primer annealing and extension: 1 cycle at 50°, 30 min; 1 cycle of 95°, 15 min; 30 cycles of 94° for 45 seconds, 55° for 45 seconds, and 72° for 2 minutes. At the end of the 30 cycles, the reaction mixture was incubated for an additional 10 min at 72°C prior to cooling to 4°C. The amplified cDNA was resolved by agarose gel electrophoresis, and the observed band of ~1.5Kb of the negative control was purified and cloned into pGEM-T easy for sequencing. Sequencing the product revealed it to differ from both the *E. californica* and *P. somniferum* CPR1 nucleotide sequences reported in WO 99/11765.

A Clonetech SMART RACE cDNA amplification kit was used to generate 5′ and 3′ RACE products from the partial *P. somniferum* cDNA generated above. Primers were designed which would anneal preferentially to the partial cDNA sequence under the selected PCR conditions by choosing regions of the partial sequence that shared minimal homology with *P. somniferum* CPR1 gene sequence. The primer sequences for 5′ and 3′ RACE are set out below.

5′ RACE primer: 5′ GGTTCTGGCATGGGTGCAGAGCATCATAGC 3′ (CNRAS1) (SEQ ID No:5)

3′ RACE primer 5′ GCGCTAGTGAATGAGAGAACACCAGCTGGTC 3′ (CNRS1) (SEQ ID No:6)

The manufacturers instructions were followed and both 5′ and 3′ RACE products of ~1.2Kb (5′ product) and 1.1Kb (3′ product) were generated. These PCR products were cloned into pGEM-T easy and sequenced. From the sequence information and the earlier sequence information from the product generated using CPR1 primers, a full length contig was constructed comprising 2816 b.p. Translation from the putative start codon produced a 698 amino acid protein. The new gene was termed CPR2. Primers were then designed to clone a CPR2 cDNA which encompassed the entire coding region and included the 5′ untranslated region (UTR). The primers used were:

5′ GATTTCAGAATTTCTCACCACAAAACCAGAGAC 3′ (CPR2_1F) (SEQ ID No:7)

5′ TCACCAGACATCACGTAGATACCTCCCATC 3′ (CNTGAAS) (SEQ ID No:8)

One step RT-PCR using a Qiagen One Step RT-PCR kit was carried out on *P. somniferum* RNA under the following conditions: 1 cycle at 50° , 30 min; 1 cycle of 95° , 15 min; 30 cycles of 94° for 45 seconds, 65° for 45 seconds, and 72° for 2 minutes. At the end of the 30 cycles, the reaction mixture was incubated for an additional 10 min at 72°C prior to cooling to 4°C. A ~ 2.4Kb product was identified by agarose gel electrophoresis and was cloned into pGEM-Teasy. Sequencing of the product revealed it to code for the full length CPR2 protein. The CPR2 nucleotide sequence (including the 5' UTR) and the translated amino acid sequences are shown in Fig. 1 (SEQ ID No:1) and Fig. 2 (SEQ ID No:2). The second ATG (underlined) in Fig. 1 commencing at nucleotide 222 is the start codon used for translation. *E. Coli* JM109 cells carrying the pGEM-Teasy plasmid with the CPR2 cDNA insert were deposited under the provisions of the Budapest treaty with the National Measurement Institute (formerly the Australian Government Analytical Laboratories) of 1 Suakin Street, Pymble, New South Wales 2073, Australia, on 30 November 2004 under Accession number NM04/43447.

EXAMPLE 2: Preparation of chimeric vector for suppression of cytochrome P-450 reductase (CPR2) activity in poppy plants.

The following steps were taken to create the silencing backbone binary vectors used to generate the vectors CPR2 hpRNA and CPR1 hpRNA.

pPLEX X002 vector (Shunmann et al, 2003) was digested with *ClaI*, blunt ended with *Pfu* polymerase and treated with Calf Intestinal Alkaline Phosphatase. The cloning vector pBC KS + containing the PdK intron (supplied by Dr N.Smith, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Canberra, Australia) was digested with *HincII* and *EcoRV* to excise the intron, and the intron fragment was ligated to the vector prepared above. The ligation mix was transformed into *E.coli* JM109 cells and clones were digested with *NotI* to screen for presence of the insert. Further screening of insert positive clones by PCR confirmed the integrity of the expression and selection cassette, and orientation screening by *BglII/XhoI* digest identified a clone that contained the intron in the correct orientation. The vector contained by this clone was designated pPLEX X002i and the steps for the construction of the vector are shown in Fig.7.

To construct CPR2 hpRNA vector, primers were designed to the 5′ end of CPR2 as there were no stretches of 22 base pair homology in this region (1-354 b.p) between CPR1 and CPR2 that could potentially cause silencing of the CPR1 gene. The primers incorporated restriction sites to facilitate cloning into pPLEX X002i. The sequences for the primers were:

5' TAGTTAACTACGTAATGGATTTCAGAATTTCTCACCACAAAACCAGAG 3'
(CPR2_1FHpaISnaBI) (SEQ ID No:9)

5' TACCTAGGCTCGAGCGGCTATAGCTGTAGTCAATATCATCAAAATCTC 3'
(CPR2_354RAvrIIXhoI) (SEQ ID No:10)

PCR of the CPR2 DNA in pGEM-Teasy (prepared in Example 1) was carried out under the following conditions: 1 cycle of 95°, 15 min; 30 cycles of 94° for 45 seconds, 65° for 45 seconds, and 72° for 2 minutes. At the end of the 30 cycles, the reaction mixture was incubated for an additional 10 min at 72°C prior to cooling to 4°C. A product of the correct size (~350 b.p) was amplified and cloned into pGEM-Teasy.

pGEM-Teasy containing the CPR2 hpRNA cDNA fragment and pPLEX X002i were both cut with SnaBI and XhoI restriction enzymes, and the CPR2 hpRNA cDNA fragment (insert) was subsequently ligated to the cut pPLEX X002i vector to form the sense arm of the hpRNA construct. JM109 cells were transformed with the ligation mix and a positive clone was selected which contained the CPR2 cDNA hpRNA sense insert. This clone was designated pPLEX X002i/CPR2 hpRNA S arm.

pPLEX X002i/CPR2 hpRNA S arm and pGEM-T easy containing the CPR2 fragment were both cut with *HpaI* and *AvrII*. The CPR2 hpRNA cDNA fragment (insert) was ligated to X002i/CPR2 hpRNA sense arm (vector) in the antisense direction to form the complete hpRNA construct. JM109 cells were transformed with the ligation mix and a positive clone was selected containing the complete CPR2 hpRNA vector.

EXAMPLE 3: Preparation of chimeric vector for suppression of cytochrome P-450 reductase (CPR1) activity in poppy plants

To construct CPR1 hpRNA vector, primers were designed to the 5' end of CPR1 as there were no stretches of 22 base pair homology in this region (1-232 b.p) between CPR1 and CPR2 that could potentially cause silencing of CPR2. The primers incorporated restriction sites to facilitate cloning into pPLEX X002i. The sequences for the primers were:

5' TAGTTAACTACGTACGGCACGAGCTTGTTAGTATCTTCTAGGGTTTG 3'
(CPR1_1FHpaISnaBI) (SEQ ID No:11)

5' TACCTAGGCTCGAGTTGAAGCTACAGTTGTGACCATAATGAAAATTGG 3'
(CPR1_232RAvrIIXhoI) (SEQ ID No:12)

PCR of the CPR1 DNA in pGEM-T was carried out under the following conditions: 1 cycle of 95°,15 min; 30 cycles of 94° for 45 seconds, 65° for 45 seconds, and 72° for 2 minutes. At the end of the 30 cycles, the reaction mixture was incubated for an additional 10 min at 72°C prior to cooling to 4°C. A product of the correct size (~230 b.p) was amplified and cloned into pGEM-Teasy.

pGEM-Teasy containing the CPR1 hpRNA cDNA fragment and pPLEX X002i were both cut with *SnaBI* and *XhoI* restriction enzymes and the CPR1 hpRNA cDNA fragment (insert) was subsequently ligated to cut pPLEX X002i vector to form the sense arm of the hpRNA construct. JM109 cells were transformed with the ligation mix and a positive clone was selected which contained the CPR1 cDNA hpRNA sense insert. This clone was designated pPLEX X002i/CPR1 hpRNA S arm.

The pPLEX X002i/CPR1 hpRNA S arm vector and pGEM-T easy containing the CPR1 fragment were subsequently both cut with *HpaI* and *AvrII*. CPR1 hpRNA cDNA fragment (insert) was then ligated to X002i/CPR1 hpRNA sense arm (vector) in the antisense orientation to form the complete hpRNA construct. JM109 cells were transformed with the ligation mix and a positive clone containing the complete CPR1 hpRNA vector was selected.

EXAMPLE 4: Transformation of *P. somniferum* cells and generation of transgenic poppy plants.

4.1: Preparation of *P. somniferum* seedlings

Seeds from the *P. somniferum* genotype CO58-34 were provided by Tasmanian Alkaloids Pty Limited, Westbury, Tasmania, Australia. The seeds were surface sterilised by washing for 30 to 60 seconds in 70% ethanol then in 1% (w/v) sodium hypochlorite solution plus 1-2 drops of autoclaved Tween-20 or Triton X at 121°C for 20 minutes with agitation. Seeds were rinsed three or four times in sterile distilled water or until no smell of bleach remained and placed in 90 x 25 mm petri dishes containing B50 medium using 0.8% Sigma Agar as the gelling agent (Gamborg et al., 1968)). The pH of the media was adjusted with 1M KOH to pH 5.6 and buffered with 10 mM MES (2 – [N-morpholino] ethanesulfonic acid). No growth regulators were added to the seed germination medium. Antibiotics (e.g., timentin and paromomycin) were added to all media after autoclaving and cooling to 55-65°C.

The petri dishes were sealed with Micropore tape and stored at 4°C for 24 to 48 hours. Seeds were germinated at 24°C in a 16 hour light-8 hour dark cycle. Hypocotyls were excised from

seedlings after 7-8 days of culture and were cut into 3 – 6 mm explants (usually 1-3 explants per seedling) for use in the transformation studies.

4.2: Transformation

The disarmed *Agrobacterium tumefaciens* strains AGL0 and AGL1 (Lazo at al., 1991) were used for facilitating transformation with the hpRNA transgenes prepared in Examples 2 and 3 in the generation of transgenic plants in which expression of the CPR1 or CPR2 gene is suppressed (designated CPR1 and CPR2 transgenic plants, respectively). *Agrobacterium* were maintained in glycerol at -80°C or on LB Agar plates plus appropriate selection media at 4°C. Fresh cultures were grown overnight at 28°C in 10 ml MG broth (Garfinkle and Nester., 1980) without antibiotics. The *Agrobacterium* suspension was diluted to approximately 5×10^8 cells ml^{-1} ($OD_{600} = 0.25$) for subsequent use.

Briefly, the 3-6 mm explants excised from the seedling hypocotyls prepared in Example 4.1 were inoculated by immersion in liquid *Agrobacterium* culture for 10-15 minutes. Explants were then transferred directly to Callusing Medium (CM) which is identical to B50 medium except that it included 1 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and usually 10 mM MES as a buffering agent. The growth regulator 2,4-D was added to the callusing media prior to the autoclaving step.

After 4 to 5 days, co-cultivation explants were washed in sterile distilled water, until the water was clear of *Agrobacterium,* blotted on sterile filter paper and transferred to CM medium, with or without MES buffering agent, containing 150 mg/l timentin and 25 mg/l paromomycin. Timentin was included to control *Agrobacterium* overgrowth. However, it would be clear to the skilled addressee that other suitable antibiotics may be used. Explants were transferred to fresh CM medium, with or without MES buffering agent, at three weekly intervals. The explants initially produced friable brownish Type I callus, and small regions of white compact embryogenic (Type II) callus, at about 7 to 8 weeks culture.

The Type II callus was transferred to B50 medium containing 150 mg/l timentin and 25 mg/l parommycin, and the culture medium replaced every three weeks. Meristemoid/embryo development occurred after one or two periods on B50 medium, and are seen from about 14 to 16 weeks total culture time. Throughout the induction, growth and germination of somatic embryos, and the development of roots and shoots, no growth regulators were added to the medium except gibberellic acid (GA3) at between 1 and 10 mg/l. Plant development typically required a further 3 months in tissue culture before shoot and root growth was sufficient to ensure successful transplantation to soil.

EXAMPLE 5: Inheritance of transgenic hpCPR2 phenotype

5.1: Testing for paromomycin resistance

Plants generated in Example 4 were allowed to self-fertilise and their seeds were collected. T_1 individuals were grown from five independent T_0 hpCPR2 plants and tested for paromomycin resistance (leaf painting) and alkaloid phenotype. The leaf painting method consisted of using a cotton bud to rub a solution of 1 or 2% paromomycin sulphate in 0.001% Tween–20 solution onto leaf surfaces. The area painted was marked with an indelible marker. Symptoms usually appeared after 4 to 5 days and consisted of necrotic collapse. Transgenic leaves expressing the *nptII* gene showed no symptoms. The results are shown in table 1.

Table 1: Results of PMN leaf painting of unselected plants.

Family	PMN Resistant	PMN Susceptible	Inferred *nptII* insertions
219-10-1	9	7	1
219-1-1	10	6	1
219-11-1	7	10	1
219-11-3	10	6	1
219-29-1	12	4	1

5.2: Thin layer chromatography

Small samples (50-100 µl) of latex were collected on a spatula tip from the cut green capsules of poppy plants and suspended in 200 µl of buffer (8.67 mn sodium dodecyl sulphate (SDS), 200 mM $NH_4H_2PO_4$ + 20% ethanol) with an unadjusted pH of approx. 4.7. The samples were vortexed then centrifuged for 4 min at 7,200g and 10 µl of supernatant of each sample was loaded onto the origin of silica gel 60 plastic backed TLC plates (Merck). The plates were developed with the solvent mixture toluene: acetone: ethanol: ammonia at a volume ratio of 40:40:6:2. Dragendorff reagent detects alkaloids, heterocyclic nitrogen compounds and quaternary amines and is prepared by dissolving 0.85 g bismuth nitrate in 40 ml deionised

water and 10 ml glacial acetic acid, and 8.0 g potassium iodide in 20 ml deionised water. Once dissolved, the two solutions are mixed together and stored in the dark until use. The reagent was sprayed onto the developed TLC plates, and the plates allowed to stand at room temperature.

The TLC results for latex collected from a number of hpCPR2 T_0 transgenic plants generated in Example 4.2 are shown in Fig. 8. As can be seen, the transgenic plants all produced morphine (M) and codeine (C) whereas oripavine (O) and thebaine (T) levels varied noticeably between plants. Compared to normal *P. somniferum* poppy plants, the latex of the hpCPR2 T_0 plants contained noticeable levels of one or more of (S)-reticuline (R), codamine (C), laudanine (L) and laudanosine (Ls). This "reticuline" phenotype is particularly noticeable in the 219-10-1 and 219-36-1 plants.

A comparison reflecting the difference in alkaloid profile of four hpCPR2 T_0 plants relative to four hpCPR1 T_1 transgenic plants is shown in Fig. 9. The figure shows that a substantially altered alkaloid profile can be obtained by suppressing the CPR2 gene compared to suppression of the CPR1 gene, reflecting the significant difference in activity and/or substrate specificity of the cytochrome P-450 reductase enzymes encoded by the CPR1 and CPR2 genes. Specifically, the latex from the hpCPR1 T_0 transgenic plants contained significant levels of morphine, codeine and thebaine, with the plant designated 218-6-2 also containing some oripavine. None of these plants contained detectable levels of (S)-reticuline or other benzylisoquinoline alkaloid characteristic of a reticuline phenotype. In contrast, in addition to accumulating morphine and codeine, the hpCPR2 T_0 transgenic plants all contained (S)-reticuline and/or other benzylisoquinoline alkaloids. More particularly, 219-10-1 was found to contain (S)-reticuline, codamine, laudanosine and laudanine, while each of 219-23-1 and 219-24-3 contained (S)-reticuline with the latter of these plants also containing codamine. Thebaine and oripavine were also observed in each of these three plants with relatively high levels of thebaine being found in 219-10-1. No (S)-reticuline was found in the hp CPR2 T_0 plant designated 219-11-5, but that plant contains codamine and laudanine.

The results for latex collected from T_1 individuals of the transgenic family 219-11-1 are shown in Fig. 10. Lanes marked 219-2-C show the results for non–transgenic *P. somniferum* T_1 individuals regenerated from culture which did not inherent the *nptII* transgene and were thereby not paromomycin (PMN) resistant. In contrast, those inheriting the transgene possessed combinations of at least some of (S)-reticuline, codamine, laudanine and laudanosine. The reticuline phenotype is strongly expressed in 219-11-1/1.

5.3: Chiral HPLC and polymerase chain reaction (PCR) analysis of alkaloid expression

HPLC analysis was used to confirm the identity of the alkaloids. For the purposes of the analysis, 0.5 µl latex samples were diluted in microcentrifuge tubes with 250 µl of buffer (0.2M ammonium phosphate + 20% ethanol at pH 4.5). The microcentrifuge tubes were briefly vortexed to ensure mixing and then centrifuged to pellet any suspended solids. An aliquot of 200 µl from each sample was mixed with an additional 250 µl to allow analysis on a Waters Binary HPLC system (HPLC pump: Waters Alliance 2690 with on-line degassing) using Waters Breeze Software (Waters Chromotography Division, Sydney, Australia) and an Alltech, Platinum C18 Rocket: Column (53 x 7mm) at a temperature of 40° C. A Waters 2487 Dual λ Absorbance Detector was used to detect peaks at 254nm. A volatile formate buffer was used for the mobile phase (2.75 ml of 90% formic acid and 3.5ml of 28% ammonia were added to 800 ml of deionised water and the pH was adjusted to 3.9 with 90% formic acid) at a flow rate of 1.5 ml/min. Alkaloids were identified by comparison of retention times with those of standards. Reticuline in the samples was identified as essentially comprising (S)-reticuline by circular dichroism.

Next, the sum of the benzylisoquinoline alkaloids reticuline (R), codamine(Cd), laudanosine (Ls) and laudanine (Ld) was determined as a percentage of the total alkaloid content of latex from *P. somniferum* hpCPR2 transgenic plants compared to latex from *P. somniferum* non-transgenic control plants and hpCPR1 transgenic plants. The results are shown below in Table 2.

Table 2: Relative Alkaloid Content

(R+Cd+Ld+Ls)/Total Alkaloids (%)	Controls (n=26)	hpCPR2 (n=33)	hpCPR1 (n=11)
Highest value	7	44	6
Mean value	1	6	1

The results show that the relative content of the benzylisoquinoline alkaloids in latex from the hpCPR1 transgenic plants was at the background level. In contrast, the level of the alkaloids was increased in the latex from the hpCPR2 transgenic plants compared to both the

control and the hpCPR1 transgenic plants, with some hpCPR2 plants exhibiting marked accumulation of the benzylisoquinoline alkaloids.

Reverse transcriptase (RT)-PCR results for six T_1 individuals of family 219-10-1 are shown in Fig. 11A to Fig. 11C. More particularly, Fig. 11A are RT-PCR results obtains using *nptII* primers showing that of the individuals tested, only 210-10-1/1 to 219-10-1/4 are transgenic. Fig. 11B are Northern blot results showing that synthesis of CPR2 mRNA is dramatically reduced in the four transgenic individuals compared to the non-transgenic plants 219-10-1/7 and 219-10-1/8. Fig. 11C shows the results of a multiplexed RT-PCR reaction obtained using both actin and *Papaver somniferum* CPR2 primers. The actin product indicates that the loadings of amplifiable RNA are comparable while the level of PsCPR2 product for the transgenic individuals indicates those plants synthesise a lower level of CPR2 mRNA than the two non-transgenic individuals.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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